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Interactions between IgM antiglobulins and IgG antinuclear antibodies. Some aspects of D-penicillamine activities

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SUMMARY

In this report we describe an *in vitro* masking action of IgM rheumatoid factor (IgM-RF) towards IgG antinuclear antibodies (IgG-ANA) which can be recovered by using D-penicillamine (DP) as an unmasking agent. The mechanism of this masking effect was elucidated by using smears of rat free nucleus as substrate, instead of the classical rat liver cryostat sections technique. It was postulated that the 'inhibition' of IgG-ANA by IgM-RF may be due to the formation of high molecular weight complexes (HMWC); this would be the same *in vivo*. Allowing the formation of HMWC which can be removed from the circulation, IgM-RF may have a protective effect by preventing or minimizing systemic lesions. Therefore, IgM-RF may be considered as a defence response against potentially noxious ANA or antigen–antibody complexes.

Induced nephropathy and the high frequency of detection of ANA observed in rheumatoid arthritis (RA) patients treated by DP may be the result of the dissociating effect of this drug on HMWC, in which the activity of pre-existing ANA is hidden when rat liver sections are used for its detection.

INTRODUCTION

Rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) are both considered to be complex-mediated autoimmune diseases, the frontiers of which are not well defined. Interesting similarities and dissimilarities are observed in the clinico-immunological profiles of RA and SLE.

SLE is a systemic disease, while RA has been described as an extravascular immune complex disease (Zvaifler, 1974). High titres of antinuclear antibodies (ANA) are detectable in the sera of SLE patients, but a high rheumatoid factor (RF) titre is not common. On the contrary, RA is characterized by high antiglobulin activity and ANA, if detected in sera, are always found in low titres. In the same patient, during the course of the disease, it is not uncommon to find a decrease of ANA titres with a corresponding increase in RF titres (personal observation). Interestingly, the reverse situation can be found in RA patients treated with D-penicillamine (DP). During DP therapy, high titres of ANA were detected in patients who had no or only low titres of ANA, whilst a marked decrease in RF activity was observed.

These considerations lead us to speculate that RF may have an 'inhibiting' effect towards ANA, and the so-called 'induced lupus' by DP may be the result of one aspect of DP action which dissociates high molecular weight complexes (HMWC) formed between RFs and pre-existing ANA.

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MATERIAL AND METHODS

In an attempt to demonstrate the above hypothesis, experiments were performed in two steps. Firstly, using the classical immunofluorescent method and rat liver cryostat sections as substrate, we studied the *in vitro* inhibition of IgG antinuclear antibodies by a monoclonal rheumatoid factor (mRF). Secondly, the recovery of ANA was demonstrated using penicillamine as a dissociating agent.

Inhibition of ANA by mRF. Complete inhibition of ANA was obtained by the formation of soluble complexes using 1·0 ml of mRF and various dilutions of four selected sera containing high titres of IgG antinuclear antibodies. The monoclonal IgM rheumatoid factor used in these experiments was obtained as described previously (Mach *et al.*, 1975), and was devoid of any antinuclear activity.

Dissociation of RF-ANA complexes. 1·0 ml of solution containing soluble complexes (RF-ANA) was mixed with 1·0 ml of an isotonic saline solution of increasing concentrations of DP (0·1–100 mg final concentration) and incubated at room temperature. ANA was studied after incubation for different lengths of time. It was demonstrated that incubation for 2 hr and 20 mg of DP represented the most suitable conditions for further experiments.

Tests for antinuclear antibodies. Two different methods are used. (1) Antinuclear antibodies were detected by the classical indirect technique using acetone-fixed rat liver cryostat sections and fluorescein-labelled goat anti-human immunoglobulins. IgG antinuclear antibodies of sera used in these experiments were previously determined by labelled goat monospecific antisera. (2) The method of using smears of acetone-fixed free nucleus obtained from rat liver homogenates as substrate was introduced in order to demonstrate the mechanism of the masking effect of IgM–RF towards IgG–ANA.

Results of immunofluorescence staining were scored 0 to +++.

TABLE 1. The detection of ANA by immunofluorescence using rat liver cryostat sections is inhibited by the presence of mRF

	Immunofluorescence staining (rat liver cryostat sections)					
	1:10	1:25	1:50	1:100	1:500	1:1000
Sera (1)	+++	+++	+++	++	+	+
Sera (1)+mRF	++	0	0	0	0	0
Sera (2)	+++	+++	+++	++	++	+
Sera (2)+mRF	++	+	0	0	0	0
Sera (3)	+++	+++	++	+	+	0
Sera (3)+mRF	+	0	0	0	0	0
Sera (4)	+++	+++	+++	++	+	+
Sera (4)+mRF	++	0	0	0	0	0

TABLE 2. Recovery of ANA by dissociating RF-ANA complexes with 20 mg penicillamine (final concentration)

	Immunofluorescence staining (rat liver cryostat sections)
Sera (1) 1:25+mRF	0
+20 mg DP	++
Sera (2) 1:50+mRF	0
+20 mg DP	++
Sera (3) 1:25+mRF	0
+20 mg DP	++
Sera (4) 1:25+mRF	0
+20 mg DP	++

TABLE 3. Dissociating action of penicillamine. Results obtained with various concentrations of pencillamine

	Penicillamine (mg/ml) final concentrations (2 hr incubation)							
	0·1	0·5	1	5	10	20	50	100
ANA (1:25) in 1·0 ml IgM-RF	0	0	0	+	+	++	+	0

TABLE 4. Recovery of ANA by dissociating RF-ANA complexes with penicillamine. Incubation time effect

	Incubation time (min)					
	10	30	60	120	180	360
ANA (1:25)+1·0 ml IgM-RF+20 mg DP	0	0	+	++	++	++

TABLE 5. Detection of ANA complexed with mRF. When nucleus smears are used as substrate, the detection of complexes ANA is not inhibited

	Immunofluorescence staining (free nucleus smears)					
	1:10	1:25	1:50	1:100	1:500	1:1000
Sera (1)	+++	+++	+++	+++	+++	++
Sera (1)+mRF	+++	+++	+++	+++	+++	++
Sera (2)	+++	+++	+++	++	++	++
Sera (2)+mRF	+++	+++	+++	++	++	++
Sera (3)	+++	+++	+++	++	++	+
Sera (3)+mRF	+++	+++	+++	++	++	+
Sera (4)	+++	+++	+++	++	++	++
Sera (4)+mRF	+++	+++	+++	++	++	++

RESULTS

The formation of soluble complexes between IgM-RF and IgG-ANA *in vitro*, strongly inhibits the detection of ANA by the classical rat liver cryostat section technique (Table 1). ANA activity could be recovered when these complexes were dissociated by incubation with DP (Table 2).

As shown by the results given in Tables 3 and 4, the dissociating effect of DP is time- and dose-dependent. Accordingly, 20 mg/ml final concentration of DP and a 2 hr incubation period were used for all experiments. In these experimental conditions, the fluorescent staining of ANA liberated from complexes by DP is not as strong as the free ANA used for reference.

This may be due either to an incomplete dissociation of soluble complexes or to a partial inhibition of ANA by the presence of DP. This second hypothesis was confirmed by our results (Table 3) which demonstrated some inhibition of ANA activity by high concentrations of DP.

When smears of free nucleus were used as substrate (Table 5), soluble complexes did not inhibit the detection of ANA and the staining obtained with complexed ANA was as bright as that obtained with free antibodies.

DISCUSSION

Mechanism of 'inhibition' of IgG-ANA by IgM-RF

The formation of soluble complexes between RF and ANA gives conflicting results in the detection of ANA when rat liver sections or smears of free nucleus are used as substrates. Our experiments led us to question the nature of the mechanism by which ANA is 'inhibited' by RF. It is believed that classical RFs are directed against antigenic determinants in the Fc region of IgG (Goodman, 1961; Glynn, 1968). Under these conditions RFs should not inhibit ANA, except in the complexes formed between RFs and ANA, the structure of IgG-ANA is so modified such that it cannot react with nuclear structures. Another possibility is represented by a structural modification of IgG antinuclear antibodies before they are complexed by RFs, and the modified molecules react with RFs by their Fab part. In these conditions IgM-RFs which inhibit ANA may be considered as agglutinating factors that possess many properties of characteristic antibodies and react with determinants on the Fab part of human IgG hidden in the intact molecule and exposed either by enzymatic digestion at low pH or when IgG acts as an antigen–antibody complex. This situation is comparable with the loss of detectable anti-tumour antibodies in some metastatic melanomas in the presence of anti-antibodies of the IgG class (Hartman, Lewis 1974). It has been demonstrated that these agglutinators frequently occur in rheumatoid sera (Harboe, Rau & Aho, 1965). These agglutinators, like classical RFs, may be produced as a result of stimulation by autologous antigen–antibody complexes in which the determinants of Fab part of IgG are revealed. It is possible that the unmasking of hidden determinants may occur *in vivo*, since it has been shown that intracellular pH during phagocytosis may be as low as 3·5 (Rous, 1925). Nevertheless, positive ANA detected by the free nucleus system do not favour the hypothesis of inhibition of ANA by IgM agglutinators reacting with the Fab determinants of IgG-ANA. The non-detection of ANA in the rat liver section system may be due not to a real inhibition by IgM-RF but rather represent a masking effect by the formation of high molecular weight complexes which do not pass easily through tissue membranes. These complexes containing ANA react only with the free nucleus. The association between RFs and ANA has been described by McCormick & Day (1963). The recovery of ANA using the rat liver sections system after the dissociation of HMWC by penicillamine would seem to support this possibility. Further studies with the Fc and Fab parts of human IgG should confirm this hypothesis. In this report, we demonstrate that monoclonal RF can mask the detection of IgG-ANA when the conventional rat liver cryostat sections method is used as substrate. It would be interesting to demonstrate the same masking effect with polyclonal rheumatoid factors which are commonly found in rheumatoid arthritis. Studies are in progress to this end in our laboratories with rheumatoid sera. Results obtained with penicillamine as a dissociating agent and free nucleus smears as substrate indicate that masked antinuclear antibodies can be found in seropositive sera (manuscript in preparation).

Biological role of IgM-RF

The results of our *in vitro* experiments demonstrating a masking effect of IgM-RF towards ANA and the recovery of antinuclear antibodies after the action of an unmasking agent, suggest various theoretical considerations concerning the role of IgM-RF in the pathogenesis of RA, SLE and other connective diseases. On the other hand, these results can explain some aspects of the biological and clinical actions of DP used in RA therapy.

The role of RFs is not well defined. Indeed, the presence of RFs alone obviously has no pathogenic role, since in many chronic infectious diseases and in some normal persons, no synovitis or other inflammation similar to that in RA is seen, even though RFs are present in the circulation. There has been much speculation about the biological role of IgM-RF. Many studies support the concept that IgM-RF may play an important role in the pathogenesis of inflammation through the formation of HMWC. Many investigations have demonstrated in rheumatoid inflammatory tissue the deposition of these complexes with free antiglobulin activities which are usually of an IgM nature in seropositive patients (Munthe & Natvig, 1970; Bonomo *et al.*, 1970; Winchester, Agnello & Kunkel, 1970). The mixed globulin complexes which are increased in size but decreased in solubility, represent an easy prey for

phagocytes which can enhance inflammation by releasing inflammatory enzymes or substances (Weissman *et al.*, 1971; Mach *et al.*, 1968; 1974).

Other observations suggest a protective effect of IgM-RF towards noxious IgG antibodies or IgG–antigen complexes. High titres of RF are not a common finding in SLE patients. On the contrary, if ANA are detected in RA patient sera, they are always found in low titres. Our findings demonstrating a masking effect of IgM-RF towards IgG-ANA through the formation of HMWC favour this hypothesis. HMWC, by reducing the penetration of noxious ANA or IgG–antigen complexes into tissue or by facilitating their elimination by phagocytes (Barnett, Bienenstock & Bloch, 1966; Rawson, Abelson & Hollander, 1965), and by reticuloendothelial system (Benacerraf, Sebestyen & Cooper, 1959; Mannik *et al.*, 1971; Weigle, 1958), can minimize or prevent systemic lesions. According to McCormick's observations, SLE patients who have positive serum RF titres tend to have less renal injuries (McCormick & Day, 1963).

Some aspects of biological actions of DP during RA therapy

The recovery of ANA from experimental soluble complexes demonstrated in this report after the *in vitro* action of DP can explain some aspects of the clinical and biological activities of this drug used in RA treatment. The clinical improvement observed in some patients may be the result of the splitting effect of DP on high molecular weight complexes into smaller molecules which do not stimulate the liberation of inflammatory enzymes or substances from phagocytes. This unmasking effect which liberates hidden ANA from complexes can explain, in part, the high frequency of the detection of ANA in the sera of patients receiving this drug (Crouzet *et al.*, 1974), and some aspects of nephropathy (Bacon *et al.*, 1976). This does not exclude other unknown mechanisms. Indeed, it has been shown that DP can induce lupus in Wilson's disease and cystinuria patients (Caille *et al.*, 1971; Boudin *et al.*, 1971; Oliver, Lieberman & de Vries, 1972). These results also suggest the presence of hidden ANA in the sera of RA and probably of SLE or other connective diseases. Studies are at present in progress and our first results would seem to corroborate this hypothesis (manuscript in preparation).

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REFERENCES

Bacon, P.A., Tribe, C.R., Mackenzie, J.C., Verrier, J.J., Cumming, R.H. & Amer, B. (1974) Penicillamine nephropathy in rheumatoid arthritis. A clinical, pathological and immunological study. *Q.J. Med.* **45**, 661.

Barnett, E.V., Bienenstock, J. & Bloch, K.J. (1966) Antinuclear factors in synovial fluid. Possible participants in the rheumatoid inclusion body. *JAMA*, **198**, 143.

Benacerraf, B., Sebestyen, M. & Cooper, N.S. (1959) The clearance of antigen-antibody complexes from the blood by the reticuloendothelial system. *J. Immunol.* **82**, 131.

Bonomo, L., Tursi, A., Trizio, D., Gillardi, U. & Dammaco, F. (1970) Immune complexes in rheumatoid synovites: a mixed staining immunofluorescence study. *Immunology*, **18**, 557.

Boudin, G., Pepin, B., Godeau, P., Vernant, J.C. & Gouerou, A. (1971) Lupus érythémateux induit par la Pénicillamine au cours d'une maladie de Wilson. *Ann. Med. Intern.* **122**, 269.

Caille, B., Harpey, J.P., Lejeune, C., Sudre, Y. & Turpin, R. (1971) Syndrome lupique induit par la D-Pénicillamine au cours d'une maladie de Wilson. Etude clinique d'une observation. *Ann. Med. Intern.* **122**, 255.

Crouzet, J., Camus, J.P., Leca, A.P., Guillien, P. & Lievre, J.A. (1974) Lupus induit par la D-Pénicillamine au cours du traitement de la polyarthrite rhumatoide. *Ann. Med. Intern.* **125**, 71.

Goodman, J.W. (1961) Reaction of rheumatoid sera with fragments of papain digested rabbit gammaglobulin. *Proc. Soc. exp. Biol. Med.* **106**, 822.

Glynn, L.E. (1968) *Clinical aspects of Immunology* (ed. by P.G.H. Gell and R.R.A. Coombs), 2nd edn. p. 848. Blackwell, Oxford.

Harboe, M., Rau, B. & Aho, K. (1965) Properties of various anti-γ globulin factors in human sera. *J. exp. Med.* **121**, 503.

Hartmann, D., Lewis, M.G. (1974) Presence and possible role of anti-IgG antibodies in human malignancy. *Lancet*, i, 1318.

McCormik, J.N. & Day, J. (1963) The association of rheumatoid factor with antinuclear factor activity. *Lancet*, ii, 554.

Mach, P.S., Kahan, A., de Mayo, C. & Delbarre, F. (1968) Etude cytochimique des ragocytes synoviaux. *C.R. Soc. Biol. (Paris)*, **162**, 604.

Mach, P.S., Auscher, C., Kahan, A., Le Go, A. & Delbarre, F. (1974) Biochemical and cytochemical studies of acid and alkaline phosphatases in synovial fluid. *Biomedicine*, **20**, 61.

Mach, P.S., Bonneton, C., Le Go, A., Piatier, D. & Delbarre, F. (1975) Soluble complexes and antiglobulin factors detection by immunofluorescence and immunoadsorption in rheumatoid sera. *Biomedicine*, **22**, 532.

Mannik, M., Arend, W.P., Hall, A.P. & Gilliland, B.C.

(1971) Studies on antigen antibody complexes. I. Elimination of soluble complexes from rabbit circulation. *J. exp. Med.* **133**, 713.

MUNTHE, E., & NATVIG, J.B. (1970) Gamma globulin complexes and anti-gamma globulin an antibodies in rheumatoid tissue and tissue eluates. *International Symposium on Immune Complex Diseases, Spoleto, Italy* 1969, p. 86. Carlo Erba Foundation, Milan.

OLIVER, I., LIEBERMAN, N.A. & DE VRIES, A. (1972) Lupus like syndrome induced by penicillamine in cystinuria. *JAMA*, **220**, 588.

RAWSON, A.J., ABELSON, N.M. & HOLLANDER, J.L. (1965) Studies on the pathogenesis of rheumatoid inflammation. II. Intracytoplasmic particulate complexes in rheumatoid synovial fluids. *Ann. Int. Med.* **62**, 201.

ROUS, P. (1925) The relative reaction within living mammalian tissues. II. On the mobilization of acid material within cells, and the reaction as influenced by the cell state. *J. exp. Med.* **41**, 399.

WEIGLE, W.O. (1958) Elimination of antigen-antibody complexes from sera of rabbits. *J. Immunol.* **81**, 204.

WEISSMAN, G., ZURIER, R.B., SPIELER, P.J. & GOLDSTEIN, I.M. (1971) Mechanism of enzyme release from leucocytes exposed to immune complexes and other particles. *J. exp. Med.* **134**, 149S.

WINCHESTER, R.J., AGNELLO, V. & KUNKEL, H.G. (1970) Gamma globulin complexes in synovial fluids of patients with rheumatoid arthritis. Partial characterization and relationship to lowered complement levels. *Clin. exp. Immunol.* **6**, 689.

ZVAIFLER, N.J. (1974) Rheumatoid synovitis. An extravascular immune complex disease. *Arthr. and Rheum.* **17**, 297.